PHOSPHORESCENCE IN BACILLUS SPORES

Lou Reinisch
Department of Physics and Astronomy, University of Canterbury,
Christchurch, New Zealand

Barry A. Swartz
Novasol, Inc., 10655 Roselle Street, San Diego, CA 92121

Burt V. Bronk
AFRL @ SBCCOM, A.P.G., MD21010-5424

ABSTRACT

The rapid classification and identification of bacteria and bacterial spores by non-invasive means remains largely unsolved. U.V. fluorescence is becoming an accepted method for detecting the presence of biological aerosols. In other studies, auto fluorescence of bacteria has been shown to be useful in identifying bacteria in a medical application. Our present work attempts to build on this approach for environmental applications. We have measured a change in the fluorescence spectra of suspensions of Bacillus bacteria between the vegetative bacteria and their spores at room temperature. Furthermore, we have measured a significant change in the spore fluorescence as the spores are dried. Upon drying, increased light emission is measured at wavelengths longer than 400 nm. We identified this emission as phosphorescence. The phosphorescence spectra have the potential to be used to aid in bacterial spore detection and identification.

INTRODUCTION

Previous work by one of the present authors[1] showed that the use of fluorescence technology can be adapted to a rapid noninvasive technique that successfully identifies the bacterial species commonly causing acute otitis media (middle ear infections) is present in an animal model. We will briefly review this application since it shows that autofluorescence of bacteria has broader potential than some have indicated.

Report Documentation Page

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 JUL 2003	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Phosphorescence In Bacillus Spores	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Department of Physics and Astronomy, University of Canterbury, Christchurch, New Zealand	

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001523., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE	**UU**	**7**	
unclassified	**unclassified**	**unclassified**			

Table 1. Bacterial pathogens and relative occurrence of children with ear infections from 1980 through 1987 in Pittsburgh, Pennsylvania		
Bacterial Pathogen	**Mean %**	**Range %**
Streptococcus pneumonia	39	27-52
Homophiles influenza	27	16-52
Branhamella catarrhalis	10	2-15
Streptococcus, Group A	3	0-11
Staphylococcus aureus	2	0-16

Five common bacteria are predominant in causing ear infections in children. In a study by Bluestone and Klein,[2] the five most common bacteria found from 1980 through 1987 are given in Table 1. In nearly all the cases, a single bacterial species was identified in the middle ear.

In the work under discussion,[1] a non-invasive technique was developed to excite and record the fluorescence of chinchillas that had been infected with one of three species of the common infecting bacteria (S. pneumoniae, H. influenzae, or S. aureus). A typical spectrum obtained in this way is shown in figure 1. Measuring the fluorescence from several animals infected with each known bacteria created a reference library.
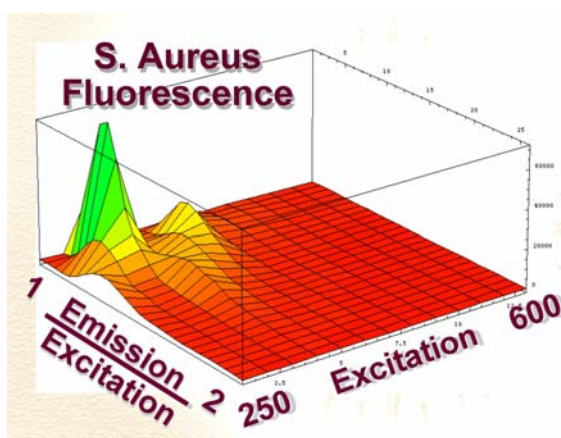

Figure 1. Typical fluorescence measured from a chinchilla infected with S aureus. The axes are Excitation wavelength from 250 nm to 600 nm; the Emission wavelength divided by the Excitation wavelength from 1.0 to 2.0 and the vertical axis is the intensity in arbitrary units.

After creating the library, we infected a second series of chinchillas and measured fluorescence from these animals in a blinded fashion. Without knowing the identity of the infecting bacterium, the measured fluorescence spectrum was fit to the library. From the fit, the identity of the bacterium was identified correctly in each instance. In Table 2, we show some of the pathogens injected in the chinchilla ears, the computer identification of the bacteria and the numerical match number that the computer fit algorithm generated when identifying the pathogen. A match number of 1.00 would be perfect match.

Table 2. The list of bacteria injected into the middle ears of chinchilla, the computer analysis the bacteria identification from the fluorescence spectrum. The Match is the linear coefficient from the least squares fit.		
Specimen	**Computer**	**Match**
1. H. influ.	H. influ.	0.76
2. H. influ.	H. influ.	0.67
3. S. aureus	S. aureus	0.63
4. S. pneumo	S. pneumo	0.98

The above results show that auto fluorescence of bacteria can be useful for a rapid and noninvasive identification of species in a medical setting. We believe that the use of this technology can be extended to environmental applications giving real time information and classification of biological particles encountered. While this is not expected to give unique information at a level comparable to what is possible with molecular recognition using such methods as PCR, it is intrinsically much faster, and we believe more specific than has been widely recognized up to now. In the present research we demonstrate that phosphorescence can be observed from bacterial spores (and other micro organisms – not shown) and gives additional parameters which can be used to classify unknown samples of environmental particles.

Dried protein powders have been shown to exhibit phosphorescence.[3] The lifetimes were in the range of 0.1 to 0.9 s. The phosphorescence exhibited the typical trident shape with peaks near 415, 450 and 465 nm. The phosphorescence is quenched when the protein is hydrated. We had previously observed this effect when looking at dry and wet samples of microorganisms[4] but did not show at the time that this was due to phosphorescence.

Tryptophan also exhibits phosphorescence when frozen with to liquid nitrogen temperatures.[5] Again, the familiar trident shape was observed with peaks near 406, 433, and 456 nm. The lifetimes are 5.5 to 6.4 s. These lifetimes are not exponential, which indicates that the lifetimes are limited by dynamic quenching.

Phosphorescence is particularly attractive to use to identify environmental particles. The longer wavelengths of the phosphorescence implies that emitted light from the unknown is shifted further away from the exciting light, making it easier to separate the exciting light from the emitted light. The phosphorescence can have cross sections that are similar to fluorescence cross sections. Additionally, if one decides that phosphorescence lifetimes are helpful in identifying a bacterial particle, the lifetimes are sufficiently long to be easy to measure.

MATERIALS AND METHODS

The B.G. spores and the B.cereus spores were prepared by standard techniques and used without further purification. The fluorescence and phosphorescence were measured on a Hitachi™ 4500 spectrofluorometer with 5 nm bandwidth (both excitation and emission) and scanning 120 nm per min. Detection was made with a photomultiplier running in a current mode (non-photon counting). The spectra were measured with excitation from 250 to 600 nm in steps of 10 nm. The emission was measured every nm from 10 nm longer than the excitation wavelength to 10 nm shorter that twice the excitation wavelength.

The spores were held by a 3M® Scotch Super Strong Packing Tape. This tape is coated with 3M® 4224 acrylic glue. This glue has a fluorescence peak that minimally overlaps with the spore fluorescence. The coated tape is held at 45 degrees in a 1 cm quartz cuvette. The suspensions of spores were suspended

in sterile saline. The saline is tested to assure that it does not fluoresce. The suspensions are measured at 90 degrees in 1 cm quartz cuvettes.

Measurements were made with the fluorometer in the "fluorescence" mode (all the emitted light was collected and measured) and in the "phosphorescence" mode (here only the emitted light approximately 1 ms after the excitation was detected). A mechanical chopper in the fluorometer was used to gate the detection system. The scan time for the phosphorescence was decreased to 60 nm per min.

All measurements were made at room temperature. There were no attempts made to control room humidity (which was low). All spectral response corrections for the fluorometer were turned off.

RESULTS

We measured the fluorescence from dried BG spores and BG spores in suspension. These spectra are shown in figure 2.
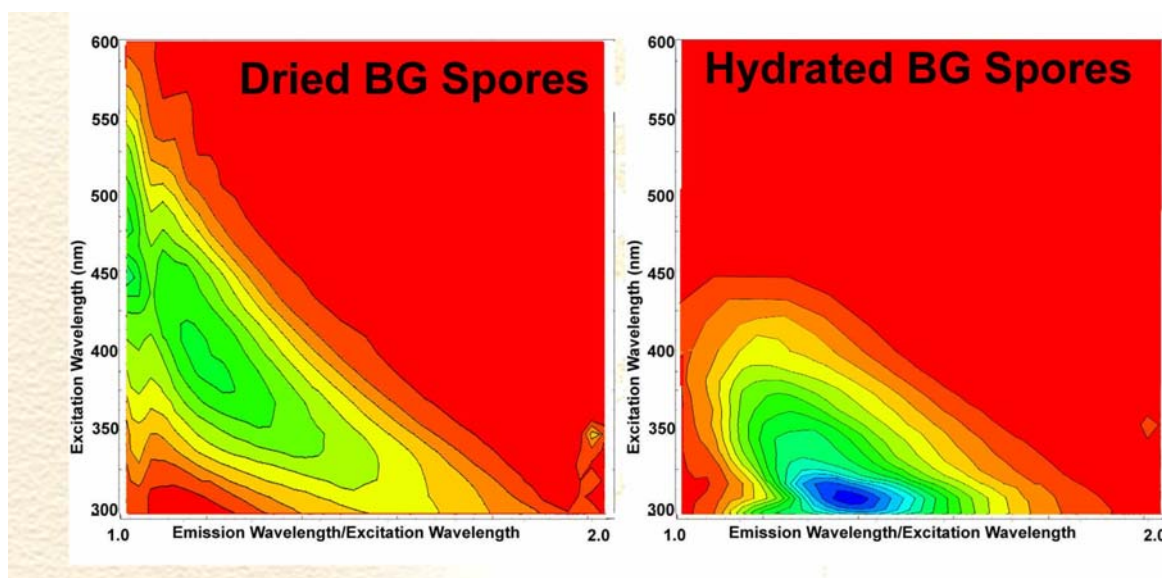

Figure 2. A comparison of the fluorescence (a mixture of fluorescence and phosphorescence) from BG spores dried and hydrated.

In figure 3, we look at the spectra measured for dried and hydrated samples at 270 and 300 nm. We compare the spectra of the dried and hydrated spores. The dried spores should contain fluorescence and phosphorescence emissions. The hydrated sample should contain only fluorescence emissions. If we subtract the emission of the hydrated spores from the dried spores, only the phosphorescence should remain. We then compared this difference with a measure of the phosphorescence using the spectrofluorometer with its internal chopper running.
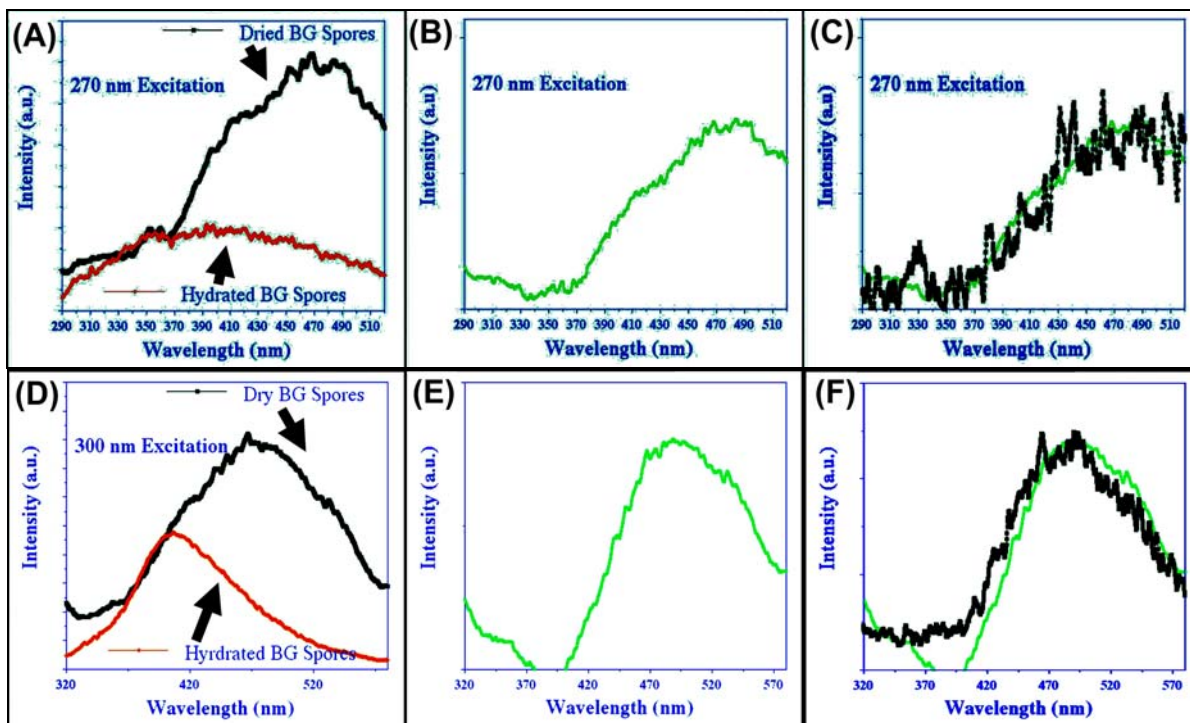
Figure 3. We show the measured emissions from wet and dried spores excited with 270 nm (A) and with 300 nm (D). We then subtracted the emission from the hydrated spores from the emission of the dried spores (B) and (E), respectively. This difference is compared with the phosphorescence measured using the spectrofluorometer and its internal chopper (C) and (F), respectively.

DISCUSSION

Radiative transitions between two states of different multiplicity are called phosphorescence. An example of such a transition is the $T_1 \longrightarrow S_0$ transition. The long lifetime of phosphorescence states, which are typically within the range of 0.1 ms to 100 s, is due to the requirement of spin reorientation and the low probability of this process. Due to the long lifetime of the state T_1, phosphorescence is especially sensitive to quenching; therefore, it is usually found at low temperatures, for example at liquid nitrogen temperature (-196°C).

Phosphorescence is a process of emission from the triplet state that is characterized by a considerably longer wavelength shift emission spectrum and the substantial lifetime of the emission process. To consider the process associated with phosphorescence in more detail, let us consider the energy level diagram in figure 4. The absorption of light causes the molecule to make the transition to the electronically excited state S_1. The processes of intramolecular relaxation and the establishment of thermal equilibrium happen very rapidly, with a lifetime on the order of picoseconds.

Deactivation of the excited singlet state, S_1, might occur prior to intermolecular relaxation, in the course of relaxation or after equilibrium has been established in the excited state. There are three possible mechanisms for the deactivation: the emission (fluorescence) with a rate coefficient k_F, the intersystem crossing (transition to the triplet state) with rate coefficient k_{ST}, or the emissionless decay with the rate

coefficient $k_d{}^S$. The transition to the triplet state is associated with an alteration in the orientation of the electron spin and is quantum mechanically forbidden. However, the spin-orbital interaction causes a mixing of the singlet and triplet wavefunctions. Thus, it is possible to make the singlet to triplet transition. The rate coefficient for the transition is sufficiently fast, $k_{ST} \sim 10^8$ s^{-1}, to allow this transition to compete with fluorescence.

As can be seen in figure 4, two types of processes control the efficiency of phosphorescence: those limiting the rate of intersystem crossing and those determining the depopulation of the T_1 state. We therefore note that the phosphorescence lifetime, τ_P, is not directly and unambiguously linked to the phosphorescence quantum efficiency, Q_P. Each of these values can change without affecting the other one.
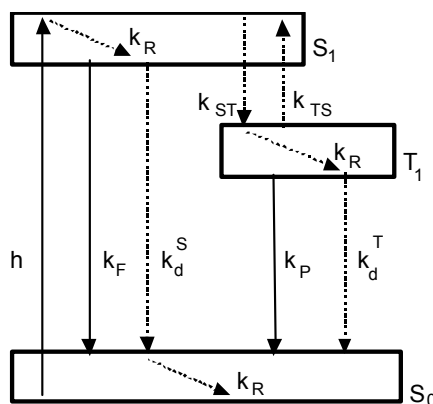

Figure 4. Electronic energy level diagram and the transitions affecting fluorescence and phosphorescence. S_0, S_1 and T_1 are the unexcited singles, excited singlet (fluorescence) and the triplet (phosphorescence) chromophore states. k_F and k_P are the rate coefficients for fluorescence and phosphorescence and $k_d{}^S$ and $k_d{}^T$ are the rate coefficients for internal conversions from the excited singlet and triplet state. The solid arrows are light absorbing and emitting transitions. The dashed arrows are dark transitions. The sloping arrows are structural relaxations with rate coefficients k_R.

The emission spectrum of dried spores is definitely shifted to the red when compared to the emission spectrum of the hydrated spores. We have assumed this red shift is due to phosphorescence that is present in the emission spectrum of the dried spores, but is dynamically quenched in the hydrated spores. This shift does not occur instantaneously, but requires an hour or more—although we did not directly measure the kinetics for this transition.

To support our hypothesis about the phosphorescence, we subtracted the hydrated emission spectrum from the dried spores and compared this difference to the measured phosphorescence and saw very good agreement. The subtraction of the two spectra is only approximate. The number of spores in the optical field was approximately equal, but definitely not identical between the two samples. Also, the dried sample was measured with 45-degree angle of scattering from spores fixed to a surface and the hydrate sample was measured at 90 degrees with the spores in suspension. The dried samples had a lot of the exciting light reflected into the detector monochromator. Thus, to make the subtraction, the two spectra should have been normalized to the number spores measured and the background subtracted. These corrections are difficult to make accurately. Similar changes in spectra we also noted for B. cereus and B. popilliae, but those data are not shown.

The phosphorescence can aid in the detection and identification of bacillus spores. The phosphorescence signal is about the same size as the fluorescence signal. Also, since the

phosphorescence is shifted to the red, it is easier to separate from the exciting light. Finally, one might be able to use the phosphorescence lifetimes to aid in identifying the source of the phosphorescence. These lifetimes are typically on the 1 ms to 1 s. They are, therefore, easier to measure than the ns lifetimes of fluorescence.

CONCLUSION

We have shown that phosphorescence exists in bacillus spores and gives a strong signal. Thus phosphorescence is another measurable parameter that can aid in real time classification of particles found in the environment.

ACKNOWLEDGEMENTS

REFERENCES

1. B.C. Spector, L. Reinisch, D. Smith, J.A. Werkhaven. Noninvasive fluorescent identification of bacteria causing acute otitis media in a chinchilla model. Laryngoscope.110, 1119-1123, (2000).
2. C.D. Bluestone, J.O. Klein, Otitis media, atelesctasis, and eustachian tube dysfunction in Pediatric Otolaryngology, Second Edition, C.D. Bluestone, S.E. Stool, and M.D. Scheetz (Eds) W.B. Saunders 321-381 (1990).
3. V.M. Mazhul, S.V. Konev, Y.S. Ermolaevm, M.A. Martynova, V.P. Nikolska, and Z.V. Prokopova. The study of equilibrium dynamics of protein structure in cell by tryptophan phosphorescence at room temperature. Biophysica (Mosc) 28:980-985 (1983).
4. B.V. Bronk., L. Reinisch. Variability of steady state bacterial fluorescence with respect to growth conditions, Appl. Spectrosc. 47:436-440, (1993).
5. J.W. Longworth, Luminescence of polypeptide and proteins, In: R.F. Steiner and I. Weinryb (Eds.) Excited states of proteins and nucleic acids. Plenum, New York. pp 319-487 (1971).